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IMMUNOCHEMICAL CHARACTERIZATION OF ANTI-ACETYLCHOLINESTERASE INHIBITORY MONOCLONAL ANTIBODIES

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SUMMARY

Monoclonal antibodies (mAbs) were prepared against native or DFP-inhibited *Torpedo californica* acetylcholinesterase and native or DFP-, MEPQ-, and soman-inhibited fetal bovine serum acetylcholinesterase. The cross reactivity of these antibodies with acetylcholinesterases from various species and their ability to inhibit catalytic activity were determined. Eight antibodies were found to inhibit catalytic activity of either *Torpedo* or fetal bovine serum enzyme. In all cases the antibodies bound to the native form of the enzymes and in some cases even to the denatured form. None of the antibodies recognized human or horse serum butyrylcholinesterase. Sucrose density gradient centrifugation of enzyme-antibody complexes provided two types of profiles, one with multiple peaks, indicating numerous complexes between tetrameric forms of the enzyme, and the other with single peaks, demonstrating complex formation within the tetrameric form. Different antibodies appeared to interact with slightly different regions, but in all cases the binding encompassed the peripheral anionic site. Decrease in catalytic activity of the enzyme was most likely caused by conformational changes in the enzyme molecule resulting from interaction with these mAbs.

Key words: Monoclonal antibodies — Anti-cholinesterase — Inhibition — Fetal bovine serum acetylcholinesterase — *Torpedo californica* acetylcholinesterase

INTRODUCTION

Monoclonal antibodies (mAbs) have been employed as molecular probes to map and investigate the surface topology of cholinesterases (ChEs) and other similar enzymes [1–4]. Those monoclonal antibodies that, upon interaction with

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enzymes, affect catalytic function by inhibition, stimulation, or other means, are of special interest. Elucidation of the structure-function correlation of ChEs can be facilitated by studying the nature of interaction of such mAbs with ChEs, since they are presumed to be directed against an epitope that either constitutes or affects the catalytic machinery of the enzyme. It can be assumed that this epitope is not the active center, since this site is located in a pocket-like conformation. This conformation was first proposed using studies with monoclonal antibodies against a synthetic peptide mimicking the sequence of the active site of fetal bovine serum acetylcholinesterase [2] and confirmed by X-ray crystallography of the enzyme from *Torpedo californica* [5]. We describe here the modulation of catalytic activity of acetylcholinesterase following the interaction with inhibitory mAbs raised against fetal bovine serum acetylcholinesterase (FBS AChE) and *T. californica* AChE. Our results indicate that although these mAbs appear to bind to a conformational epitope located in a region remote from the catalytic site that is at or near the peripheral site, the conformational changes caused in the molecule modulate the catalytic mechanism.

MATERIALS AND METHODS

Mice were immunized with a mixture of 5.6 S hydrophobic dimer and asymmetric (17+13) S forms of *T. californica* AChE, native FBS AChE, DFP-inhibited FBS AChE, soman-inhibited FBS AChE, or MEPQ-inhibited FBS AChE. Details of the fusions have been previously published [1,3,6]. Enzymes from fetal bovine, fetal equine, human sheep, pig, rabbit, goat, and horse sera were isolated using the method of De La Hoz et al. [7] using sera from commercial sources. Inhibition of serum enzymes was assessed by a microplate adaptation of the Ellman assay [8]. Recognition of serum enzymes by antibodies was measured by ELISA, using purified enzymes to coat the plates and a horseradish peroxidase-conjugated anti-mouse antibody to detect binding. Sucrose density gradients were used to estimate the size and the nature of complexes formed between monoclonal antibodies and FBS AChE. Edrophonium, an esteratic-site ligand, and propidium, a peripheral anionic-site ligand, were employed to determine whether binding of antibodies interfered with the hydrolysis of acetylthiocholine by AChE. Prevention or reduction of DFP binding and displacement of DFP by TMB₄ in enzyme/antibody complexes were also assessed.

RESULTS AND DISCUSSION

A total of eight inhibitory monoclonal antibodies were produced, two (2C8, 7G4) from mice immunized with *Torpedo* AChE, one each from fusions with native (13D8) and DFP-inhibited FBS AChE (25B1), and four (2A1, 4E5, 5E8, and 6H9) from a fusion with MEPQ-FBS AChE as the immunogen (Table I). No inhibitory antibodies resulted from a soman-FBS AChE immunization, although immunization and fusion were done under identical conditions as the MEPQ-AChE fusion.

None of the antibodies showed any cross-reactivity in inhibition patterns, i.e.,

TABLE I
IMMUNOCHEMICAL CHARACTERIZATION OF ANTI-ACETYLCHOLINESTERASE MONOCLONAL ANTIBODIES

Antibody isotope	Enzyme antigen	Inhibition of AChE activity, % ^a	[³ H]DFP binding to mAb:AChE, % ^b	³ H activity after TMB ₄ , % ^c	Est. MW of mAb:AChE complex ^d	Effect of edrophonium/propidium ^e
13D8, IgG ₁	FBS AChE	84	14	0	≥ 860 000	0/0
25B1, IgG ₁	DFP-FBS AChE	>98	7	5	≥ 860 000	0/+
2A1, IgG ₁	MEPQ-FBS AChE	92	26	67	≥ 860 000	+/+
4E5, IgG ₁	MEPQ-FBS AChE	>98	10	59	580 000	0/+
5E8, IgG _{2a}	MEPQ-FBS AChE	>98	17	49	430 000	+/+
6H9, IgG _{2b}	MEPQ-FBS AChE	>98	5	75	430 000	0/+
None	FBS AChE	0	100	0	—	—
2C8, IgG ₁	<i>Torpedo</i> AChE	93	63	80	≥ 860 000	N.D.
7G4, IgG ₁	<i>Torpedo</i> AChE	95	48	85	≥ 860 000	N.D.
None	<i>Torpedo</i> AChE	0	100	0	—	—

^aEqual amounts of either FBS AChE or *Torpedo californica* 11S AChE and appropriate amounts of monoclonal antibody were incubated at room temperature for 24 h and assayed for residual enzyme activity.

^bEnzyme:antibody complexes formed after 24 h incubation (see above) were incubated for an additional 24 h with a 2-fold molar excess (to enzyme) of [³H]DFP. Enzyme:mAb:[³H]DFP was separated from free [³H]DFP by Bio-Rad P-6 gel filtration chromatography. Radioactivity eluting with enzyme alone was used as the 100% control to compare with labeling of various enzyme:mAb complexes.

^cFBS AChE and *Torpedo* AChE were prelabeled with [³H]DFP (2-fold molar excess) by incubating at room temperature for 24 h. Enzyme:[³H]DFP complex was isolated by gel filtration on Bio-Rad P-6 columns and incubated with mAb for 24 h, followed by incubation for 48 h with 1 mM TMB₄. Separation by P-6 column chromatography allowed determination of the amount of radioactivity bound to enzyme or enzyme:mAb.

^dEstimated molecular weight of AChE:mAb complexes formed, as determined by sucrose density gradient centrifugation (5–20%) using molecular weights of 280 000 for FBS AChE (tetrameric form) and 150 000 for antibody.

^eThe relative effect of edrophonium (upper symbol) and propidium (lower symbol) on the rate of acetylthiocholine hydrolysis; zero, no effect; +, change in rate of inhibition by mAb.

anti-FBS AChE antibodies did not inhibit *Torpedo* enzyme and vice versa. Four mAbs (25B1, 4E5, 5E8, and 6H9) inhibited FBS AChE greater than 98% at an enzyme:mAb ratio of 1:1 – 1:5. Antibodies 13D8 and 2A1 partially inhibited FBS AChE, 84% and 92%, respectively; antibodies 2C8 and 7G4 partially inhibited *Torpedo* AChE, 93% and 95%, respectively.

None of the antibodies recognized or inhibited butyrylcholinesterase from either human or horse serum. As determined by ELISA, all anti-FBS AChE antibodies recognized the cholinesterase from the serum of sheep, rabbits, and goats, that was identified as acetylcholinesterase by use of the specific inhibitors BW284C51 and iso-OMPA [9]. None of these antibodies recognized enzyme isolated from pig, horse, fetal equine, or human sera, identified as butyrylcholinesterase by the same inhibitors. All anti-FBS AChE antibodies recognized epitopes on recombinant human AChE, although the binding was minimal. Five of the six anti-FBS AChE antibodies were able to bind to some extent to reduced, denatured, and alkylated FBS AChE.

Sucrose density gradient centrifugation of (tetrameric) enzyme-antibody complexes revealed that some anti-FBS AChE mAbs (4E5, 5E8, 6H9) produced complexes of a discrete size, while others (25B1, 13D8, 2A1) formed multimeric complexes. The distance between epitopes or their orientation on the surface of catalytic subunits of the tetrameric form of FBS AChE for the first group of antibodies appears to be such that the complex is composed of only a single tetramer and either one or two antibody molecules. For mAbs 5E8 and 6H9, the orientation of the first bound antibody molecule appears to prevent the binding of a second antibody molecule, while in the case of mAb 4E5, the epitopes seem to be oriented so that each tetramer can bind two antibody molecules. For the second group of mAbs, those forming multimeric complexes, the distance between their epitopes must be different or the location must be such that the antibodies can bridge between tetramers, since the complexes appear to be formed of more than one tetramer and more than two antibody molecules.

Inhibition reactions measuring decrease in FBS AChE activity following complexation with the six anti-FBS AChE mAbs were somewhat different for each antibody. However, propidium was found to be somewhat more effective than edrophonium in inhibiting the complexation reaction, suggesting that these antibodies bind in the vicinity of the peripheral anionic site of the enzyme.

Five of six anti-FBS AChE mAbs effectively interfered with the binding of [3 H]DFP to FBS AChE after enzyme:antibody complexes were formed. When FBS enzyme was inhibited by anti-FBS AChE monoclonal antibodies, the binding of [3 H]DFP to enzyme was markedly retarded (only 5 – 17% bound after 24 h). Antibody 2A1 was less efficient at retarding DFP binding, allowing 26% to be bound, even though enzyme inhibition was 92%. The anti-*Torpedo* AChE antibodies only partially prevented [3 H]DFP binding to the *Torpedo* enzyme. For complexes of two mAbs, 13D8 and 25B1, with FBS AChE and [3 H]DFP, TMB₄ was effective at dissociating [3 H]DFP. For the remaining antibodies and their complexes, TMB₄ only partially dissociated the DFP (49 – 85%). These results suggest that binding of all anti-FBS AChE mAbs to a region of the enzyme which is remote to the catalytic site affects acylation/phosphorylation. The dissociation

of DFP by TMB₄ from the complex with mAbs 25B1 and 13D8 suggests that in these two cases the deacylation/phosphorylation mechanism of the enzyme is still functioning, which is not true with the other mAbs.

The results presented here indicate that the change in conformation of AChE caused by the complex formation between anti-AChE mAbs and the enzyme in a region remote to the active site affects the catalytic mechanism of the enzyme.

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REFERENCES

- 1 B.P. Doctor, S. Camp, M.K. Gentry, S.S. Taylor and P. Taylor, Antigenic and structural differences in the catalytic subunits of the molecular forms of acetylcholinesterase, *Proc. Natl. Acad. Sci., U.S.A.*, 80 (1983) 5767-5771.
- 2 R.A. Ogert, M.K. Gentry, E.C. Richardson, C.D. Deal, S.N. Abramson, C.R. Alving, P. Taylor and B.P. Doctor, Studies on the topography of the catalytic site of acetylcholinesterase using polyclonal and monoclonal antibodies, *J. Neurochem.*, 55 (1990) 756-763.
- 3 Y. Ashani, M.K. Gentry and B.P. Doctor, Differences in conformational stability between native and phosphorylated acetylcholinesterase as evidenced by a monoclonal antibody, *Biochemistry*, 29 (1990) 2456-2463.
- 4 Y.A. Ashani, A. Bromberg, D. Levy, M.K. Gentry, D.R. Brady and B.P. Doctor, Changes in the catalytic activity of acetylcholinesterase upon complexation with monoclonal antibodies, in: J. Massoulié, F. Bacou, E. Barnard, A. Chatonnet, B.P. Doctor and D.M. Quinn (Eds.), *Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology*, American Chemical Society, Washington, DC., 1991, pp. 235-239.
- 5 J.L. Sussman, M. Harel, F. Frolov, C. Oefner, A. Goldman, L. Toker and I. Silman, Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science*, 253 (1991) 872-879.
- 6 M.K. Gentry, E.A. Henchal, J.M. McCown, W.E. Brandt and J.M. Dalrymple, Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies, *Am. J. Trop. Med. Hyg.*, 31 (1982) 548-555.
- 7 D. De la Hoz, B.P. Doctor, J.S. Ralston, R.S. Rush and A. D. Wolfe, A simplified procedure for the purification of large quantities of fetal bovine serum acetylcholinesterase, *Life Sci.*, 39 (1986) 195-199.
- 8 G.L. Ellman, K.D. Courtney, A. Andres, Jr. and R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochem. Pharmacol.*, 7 (1961) 88-95.
- 9 L. Austin and W.K. Berry, Two selective inhibitors of cholinesterase, *Biochem. J.*, 54 (1953) 695-700.

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